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INHIBITION OF BACTERIAL GROWTH BY INDOLEACRYLIC ACID AND ITS RELATION TO TRYPTOPHAN; AN ILLUSTRATION OF THE INHIBITORY ACTION OF SUBSTANCES CHEMICALLY RELATED TO AN ESSENTIAL METABOLITE.

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WHEN studying the production of indole from tryptophan by suspensions of *Bact. coli*, it was noted by Fildes (1938) that the growth of *Bact. coli* on an ammonium lactate medium was inhibited by indoleacrylic acid but not by various other indole derivatives in the concentrations tested. This observation has been pursued in the light of the theory of chemotherapy discussed by Fildes (1940*b*).

PROCEDURE.

The general procedure was similar to that used by Fildes (1940*a*). Three strains of test bacteria were used, *Bact. coli*, *Bact. typhosum* and the same strain trained to grow on NH_3-lactate. For *Bact. coli* and the trained strain of *Bact. typhosum*, the NH_3-lactate medium (Fildes, 1940*a*) was used, for *Bact. typhosum*, untrained, the amino-acid mixture of Gladstone (1939), together with M/80 glucose as a source of energy.

In the case of *Bact. coli* and the trained *Bact. typhosum* the inoculum was derived from a previous culture in the same medium. The untrained typhoid was taken from agar. Dilutions were made in the medium used. The number of organisms added per tube was 1000 or 10,000. The size of inoculum was not of so much importance as in the case of streptococcus and sulphanilamide (Woods, 1940), neither was the age of the culture. This varied from 1 to 4 days.

EXPERIMENTS WITH *BACT. COLI*.

In preliminary experiments dilutions of indoleacrylic acid from M/1000 downwards were compared for inhibitory action with certain indole derivatives.

With indoleacrylic acid inhibition of growth was complete for 3 days at least, in concentrations ranging from M/1000 to M/8000. M/16,000, however, grew slightly on the third day. No inhibition occurred with indoleacetic acid or indolealdehyde, but during the first day and in the higher concentrations indolecarboxylic acid inhibited slightly and indolepropionic rather more.

Indoleacrylic acid had therefore a much greater inhibitory action than had the other derivatives tested. For the reasons that (1) indoleacrylic acid is very closely related to tryptophan, (2) it is capable of stimulating the production of the tryptophan-indole enzyme of *Bact. coli* (Fildes, 1938), and (3) it leads to the production of indole in a culture of *Bact. coli* as does no other derivative (Fildes, 1938), it was clear that indoleacrylic acid has some closer relation to tryptophan and its enzymes than have the other derivatives, and it was thought that the inhibition of growth might be due to this association and result from interference with an enzyme engaged in the metabolism of tryptophan. Tryptophan is an " essential metabolite " in the sense of Fildes (1940*b*), and it was possible that indoleacrylic acid might interfere with it in a manner similar to that by which sulphanilamide interferes with p-aminobenzoic acid (Woods, 1940). Experiments were therefore carried out to test the power of tryptophan and other substances to counteract the inhibition of *Bact. coli* by indoleacrylic acid. Experiment 1 shows that tryptophan, which has no growth stimulating action itself, counteracts the inhibition by indoleacrylic acid. The growth which follows the addition of tryptophan is proportional in mass to the tryptophan added, and there is no quantitative relationship

EXPERIMENT 1.—*Inhibition of* Bact. coli *by Indoleacrylic Acid and Counteraction by Tryptophan.*

NH_3-lactate medium; 10,000 organisms.

Tube.	Indoleacrylate. M/-.	Tryptophan. M/-.	Growth in hours or days.			
			19.	26.	44.	3–9.
1	0	0	tr.	+	++	+++
2	1000	0	0	0	0	0
3	,,	1,000	0	tr.	+	+++
4	,,	5,000	0	tr.	+	++
5	,,	25,000	0	tr.	>tr.	+
6	,,	125,000	0	tr.	tr.	tr.
7	5000	0	0	0	0	0
8	,,	1,000	tr.	+	++	+++
9	,,	5,000	tr.	+	+	++
10	,,	25,000	tr.	>tr.	>tr.	+
11	,,	125,000	tr.	tr.	tr.	tr.
12	0	1,000	tr.	+	++	+++
13	0	5,000	tr.	+	++	+++
14	0	25,000	tr.	+	++	+++
15	0	125,000	tr.	+	++	+++

Signs roughly proportional to the mass of growth; tr. = trace.

between the inhibitory concentration of indoleacrylic acid and the countering concentration of tryptophan, in that the amount of growth produced by any dilution of tryptophan is the same with both concentrations of indoleacrylic acid. This counteraction was not shown in the presence of M/5000 indoleacrylic acid by the derivatives already mentioned, nor by indolepyruvic acid, indole ethylamine, skatole or indole itself. That the effect is specific to tryptophan is also indicated by the fact that a hydrolysate of gelatine, though stimulating growth, has no activity.

The result of Experiment 1 is as would be expected if it is assumed that indoleacrylic acid inhibits the formation of tryptophan and not its utilization. Then a quantitative relationship between it and indoleacrylic acid would not be expected, and the smallest quantity necessary to give growth should reverse the inhibition. This is the case.

EXPERIMENTS WITH *BACT. TYPHOSUM*.

Bact. typhosum will not grow on the simple NH_3-lactate medium, but requires a number of amino-acids including tryptophan (Fildes, Gladstone and Knight, 1933). An amino-acid medium, however, in which tryptophan is present shows no inhibition by M/1000 indoleacrylic acid, even when the tryptophan is reduced to 10^{-6} M. Indole was therefore used to replace tryptophan, since it has been shown (Fildes, 1940*c*) that growth can be obtained in this way.

Experiment 2 was carried out to determine whether indoleacrylic acid had any inhibitory action when indole was used as the growth factor in place of tryptophan. It is now found that indoleacrylic acid is inhibitory to *Bact. typhosum* in the same order of concentration as with *Bact. coli*, and that the inhibitory titre has no relation to the concentration of indole added. Experiment 3 shows the relation of tryptophan to this inhibition in the presence of a constant concentration of indole. It is seen that tryptophan completely counteracts the inhibition by indoleacrylic acid, that the amount of growth is roughly proportional to the amount of tryptophan, and that there is no relation quantitatively between the tryptophan and the indoleacrylic acid. The result is thus the same as with *Bact. coli*. None of the indole derivatives have had a similar counter effect in the concentrations tested.

As in the case of *Bact. coli*, therefore, the result of this experiment is in accordance with expectation if it is assumed that in the presence of indoleacrylic acid *Bact. typhosum* is unable to achieve the synthesis of tryptophan.

It has, however, been shown (Fildes, 1940*c*) that the synthesis of tryptophan by some bacteria can be separated into two stages, NH_3 or amino-N$\longrightarrow$ indole$\longrightarrow$tryptophan, and since in the typhoid experiments preformed indole is present, it follows that the failure of synthesis must be in the second stage, indole$\longrightarrow$tryptophan.

EXPERIMENTS WITH A TRAINED *BACT. TYPHOSUM*.

It has been shown by Fildes, Gladstone and Knight (1933) that *Bact. typhosum* can be trained to grow on a simple NH_3-lactate medium, and it has

EXPERIMENT 2.—*Inhibition of* Bact. typhosum *by Indoleacrylic Acid.*

Amino-acid-glucose medium. 10,000 organisms. The negative control (tube 1) incubated in a separate air-CO_2 jar, otherwise growth takes place with indole volatilized from the other tubes.

Tube.	Tryptophan. M/-.	Indole. M/-.	Indole-acrylate. M/-.	Growth in hours or days.		
				25.	46.	3.
1	0	0	0	0	0	0
2	100,000	0	0	+	++	++
3	0	4,000	0	0	++	++
4	0	,,	4,000	0	0	0
5	0	,,	20,000	0	0	+
6	0	,,	100,000	0	++	++
7	0	20,000	0	tr.	++	++
8	0	,,	4,000	0	0	0
9	0	,,	20,000	0	0	+
10	0	,,	100,000	0	++	++
11	0	100,000	0	tr.	++	++
12	0	,,	4,000	0	0	0
13	0	,,	20,000	0	0	+
14	0	,,	100,000	tr.	++	++

EXPERIMENT 3.—*Counteraction of Inhibition of* Bact. typhosum *by Tryptophan.*

Amino-acid-glucose medium. 10,000 organisms. M/25,000 indole present in all tubes.

Tube.	Indoleacrylate. M/-.	Tryptophan. M/-.	Growth in hours or days.		
			24.	44.	3.
1	0	0	+	++	++
2	1,000	0	0	0	0
3	,,	25,000	+	++	++
4	,,	125,000	|	|	|
5	,,	625,000	↓	↓	↓
6	,,	3,125,000	tr.	tr.	tr.
7	5,000	0	0	0	0
8	,,	25,000	±	++	++
9	,,	125,000	|	|	|
10	,,	625,000	↓	↓	↓
11	,,	3,125,000	tr.	tr.	tr.
12	25,000	0	0	0	tr.
13	,,	25,000	±	++	++
14	,,	125,000	|	|	|
15	,,	625,000	↓	↓	↓
16	,,	3,125,000	tr.	±	+

been concluded that this training results in a development of a power to synthesize amino-acids from NH_3 in particular tryptophan. It is now further concluded more exactly that it is a case of developing a power to synthesize indole from NH_3, thus making the synthetic power of the organism equivalent to that of *Bact. coli*.

The experiments were repeated, using this organism on the simple NH_3-lactate medium. The main results relating to inhibition by indoleacrylic acid and counteraction by tryptophan were the same as in the previous experiments with *Bact. coli*.

DISCUSSION.

In Wood's (1940) work on the inhibition of growth by sulphanilamide and the counter effect of the then hypothetical essential metabolite p-aminobenzoic acid. it was found that there was a strict quantitative relationship between the inhibitor and the metabolite, in that twice the concentration of the former required twice the concentration of the latter to counteract it. He adopted the view that these two substances were competing for an enzyme which normally was concerned with the metabolism of p-aminobenzoic acid, and preferred the idea that the metabolism concerned was the further utilization of p-aminobenzoic acid rather than its synthesis. If it were a case of synthesis it would not be expected that the inhibitor should have a quantitative relation with the product p-aminobenzoic acid but with a precursor. Further, the addition of p-aminobenzoic acid just sufficient for the needs of the organism should cause growth in spite of the presence of the inhibitor.

In the case of tryptophan and indoleacrylic acid, the addition of a trace of tryptophan does cause growth in spite of the inhibitor, and therefore on this argument the action of the inhibitor is more likely to be concerned with inhibition of the synthesis of tryptophan. Further, there is a complete absence of the quantitative relationship which Woods noted, again suggesting that the inhibition is exerted on the synthesis of tryptophan. There should be a quantitative relationship between indoleacrylic acid and a precursor of tryptophan. It is now known that indole is such a precursor, but again there is no relation between indole and indoleacrylic acid. The precursor assumed to exist must be situated in the synthetic series between indole and tryptophan rather then below indole, because otherwise indole would counteract the effect of indoleacrylic acid in the same way as does tryptophan. A conclusion based on Woods' argument must, therefore, assume an intermediate substance X in the synthesis indole $\longrightarrow$ tryptophan. Indoleacrylic acid may compete with this substance for the enzyme which completes the stage substance $X \longrightarrow$ tryptophan.

It must, however, be noted that variation in the concentration of indole —the precursor of the hypothetical substance X—should have the effect of varying the concentration of X and so varying the necessary concentration of inhibitor, if the effect of this is by competition with X. This is not, however, demonstrably the case. Either, therefore, the blocking by indoleacrylic acid of the enzyme concerned with synthesizing tryptophan from X is irreversible, or else it is impossible, for technical reasons, to reach the concentration of

indole (and thus of *X*) required to reverse the inhibition. In concentrations higher than M/4000, indole is found to be inhibitory of growth.

In the case of *Bact. coli*, since all the experimental results are similar to those obtained with *Bact. typhosum*, it is assumed that the synthesis of tryptophan by this organism also proceeds through indole, but that *Bact. coli* differs from *Bact. typhosum* in being able to synthesize indole from NH_3. In this case also it is concluded that indoleacrylic acid blocks the utilization of a precursor *X* in the final stage of synthesis to tryptophan.

The results of these experiments are in general accord with the hypothesis of inhibition by substances chemically related to an essential metabolite discussed by Fildes (1940*b*), the hypothesis being based on the work of Woods (1940). In Woods' example it was argued that sulphanilamide competed with p-aminobenzoic acid for the services of an enzyme which utilized p-aminobenzoic acid further in an unknown manner ; in the present case it is supposed that a substance *X* occupies the position of p-aminobenzoic acid, and the further utilization which is prevented by indoleacrylic acid has to do with the synthesis of tryptophan.

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Note (*September*, 1941).—The object of this paper is to support a hypothesis (Fildes, 1940*b*) relating to one form of chemotherapeutic effect. Other illustrations have been published by McIlwain (1940, 1941).

The hypothesis depends largely upon the supposition that p-aminobenzoic acid, a substance which was not known to have biological interest before the work of Woods (1940), is in fact an essential metabolite "not only for bacterial cells but also for animal" (Fildes, 1940*b*).

This supposition has now been shown to be correct in the case of bacteria by Rubbo and Gillespie (1940), and in the case of animals by Ansbacher (1941) and Martin and Ansbacher (1941).

This mode of action of antibacterial substances chemically related to essential metabolites may thus be said to have general application, since it is demonstrable with four different classes of compound (Woods, 1940 ; McIlwain, 1940, 1941 ; and Fildes in the present paper).

SARCOMA PRODUCED BY SUBCUTANEOUS INJECTIONS OF OVERHEATED COTTON-SEED OIL INTO MICE.

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BURROWS, Hieger and Kennaway (1932) announced that fats commonly used as vehicles for subcutaneous injection were under investigation as far as the production of tumours of the connective tissue is concerned. Three tumours occurred in a group of ten control mice injected with unheated lard in Burrows' (1932) experiments, but the author was suspicious that some confusion of animals or substances injected had occurred. Tumours did not arise in any other group of the injected mice, and the positive result could not be reproduced after complete precautions had been taken.

Peacock (1933) injected fowls with a solution of dibenzanthracene in lard into the right breast, and with lard only into the left breast. Tumours were observed at both sites.

Andervont (1934) found no new growth in 190 control mice injected with lard alone after 189 days.

Barry and Cook (1934) described tumour-like tissue in 2 of 10 rats following subcutaneous injection of lard after 385 and 462 days. The mass consisted of necrotic matter, granulation tissue, and some spindle-celled areas which were not considered to be malignant.

The experiments of Burrows, Hieger and Kennaway (1936) yielded not a single tumour in 216 mice after the injection of fats, fatty acids, unsaponifiable materials and liquid paraffin. In rats, however, the injection of lard alone resulted in 5 spindle-celled tumours out of 107 rats. Three more tumours were observed, one after the injection of lard with oleic acid, another after the injection of lard with theelin, and another after the injection of olive oil with arsenious acid. More than a year elapsed before the tumours appeared. Injections of lard, heated at 340–360° C. for two hours in a stream of nitrogen gave rise to two spindle-celled tumours in rats after 509 and 608 days. No tumours were obtained in mice. Three peritoneal tumours have been observed after the intraperitoneal injection of olive oil, but the authors consider it unsafe to assume that the tumours were the result of the injection.

Roffo (1938, 1939) described papillomata, adenocarcinomata and sarcomata

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of the stomach, and mesenteric sarcomata, obtained by the feeding of vegetable and animal fats heated at 350° C. for half an hour.

Domagk (1939) attempted to elicit gastro-intestinal tumours in mice by adding various apparently unheated fats to the diet. Only a few mice responded with tumours or glandular hyperplasia. These lesions were not found in the controls.

Beck and Peacock (1941) found that fats, heated under conditions that might occur in domestic cooking (repeatedly heated at 200–220° C. for a total of 12 hours), did not produce carcinoma or sarcoma of the stomach, but interfered with the metabolism of vitamin A and produced papillomata, bordering on malignancy, as a result of vitamin A deficiency, in rats fed on a diet which was adequate for controls, but not rich in vitamin A. The effect of overheated fats, as in the experiments of Roffo, is now under investigation.

Mice have been chosen for the injection of overheated cotton-seed oil, because they do not respond with the production of sarcoma as easily as rats do.

EXPERIMENTS.

Three groups, each containing 12 mice, were subcutaneously injected with 0·5 c.c. cotton-seed oil into the right flank.

In Group 1 the cotton-seed oil was heated at 340–360° C. for an hour, in the presence of air in the fume cupboard.

In Group 2 the cotton-seed oil was repeatedly heated at 200–220° C. for a total of 12 hours.

In Group 3 unheated cotton-seed oil was injected.

A new syringe and needle were used for the injections, to be sure that the cotton-seed oil was not contaminated with carcinogenic substances.

RESULTS.

Only those mice which survived the latent period of the earliest tumour (414 days) have been taken into account.

In Group 1 six mice survived. In every mouse examined post-mortem, a cyst was found containing a brown liquid similar to the overheated cotton-seed oil injected. The cyst was palpable in vivo. The first tumour, (Mouse 1866) appeared at the site of injection 414 days after the start of the experiment, and seemed to arise at the cyst wall. The mouse was killed 43 days later, by which time the tumour had penetrated into the peritoneum. Histological examination by my colleague, Dr. L. Woodhouse Price, revealed a spindle-celled sarcoma with an intercellular reticulum. The second tumour appeared at the site of injection 538 days after the start of the experiment (Mouse 1869). The tumour was very close to a similar cyst containing brown fluid, and proved, histologically, to be a spindle-celled sarcoma with a fine reticulum. No secondaries were observed in either mouse, and grafts have not been undertaken.

In Group 2 three mice only survived. A cyst was usually found containing the injected cotton-seed oil, except in the mice which died after twelve months. No tumours occurred. The small number of surviving mice makes it necessary to repeat the experiment.

In Group 3 ten mice survived, and six lived for more than 560 days. Neither cysts nor tumours were observed in this group.

DISCUSSION.

Before injection a sample of the overheated cotton-seed oil (340–360° C.) and of the repeatedly heated cotton-seed oil (200–220° C.) was spectroscopically examined. The spectrum showed only a general fluorescence and no bands, proving that none of the known carcinogens was present, and proving also that no contamination with carcinogens could have occurred. The heated cotton-seed oils, after having been purified chromatographically, contained a substance which gave a red colour when added to antimony trichloride. The colour was different from the red of the cholestadiene reaction.

Besides the production of sarcomata by overheated cotton-seed oil, three facts are of interest :

First, the great persistence of the depot of overheated cotton-seed oil. Second, the nearness of the tumour to the depot of overheated cotton-seed oil. Third, the long latent period.

These three facts can be correlated to each other.

Peacock (1933) described the topical conditions of tumour and tar depot in fowls. The tumour never arose in immediate contact with the main tar depot, but at some distance from the smaller depots in the direction of the lymph stream.

Peacock and Beck (1938) observed the same fact for benzpyrene tumours, and suggested that the tumour occurred at a point at some distance from the depot where the optimum rate of absorption was maintained for an optimum time. It seems obvious that the sarcoma-producing substance acts while it is being absorbed. In the case of overheated cotton-seed oil, the tumour starts very near the depot ; this suggests that, either the rate of absorption is very slow, coinciding with the persistence of the depot, or that the concentration of the tumour-producing substance in overheated cotton-seed oil is very low.

The long latent period can also be explained by the foregoing facts. Assuming that the tumour-producing substance acts on its way to absorption, the process of inducing sarcomata appears similar to carcinogenesis as a result of painting. In the latter case, the carcinogen is applied every few days. In the case of sarcomagenesis, we have a more continuous stream of tumour-producing substances being absorbed through the tissues. We know that a dilution of the carcinogen in painting experiments results in a prolongation of the latent period. It seems justifiable to conclude, as far as sarcomata are concerned, that a slow rate of absorption or a low concentration of the tumour-producing substance brings about the conditions for a long latent period.

SUMMARY.

(1) Subcutaneous injection of overheated cotton-seed oil (340–360° C.) produced sarcomata in two of twelve mice, at the site of injection, after 414 and 538 days.

(2) Repeatedly heated cotton-seed oil (200–220° C.) and unheated cotton-seed oil did not produce tumours.

(3) Secondary findings : (*a*) A long persistent depot of overheated cotton-seed oil, (*b*) nearness of the tumour to this depot, and (*c*) a very long latent period, are correlated to each other.

I am indebted to Dr. Peacock for his interest in the work, to Dr. L. Woodhouse Price for the histological examination of the tumours, and to Mr. A. H. M. Kirby for the spectroscopic examination and the chromatogram.

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THE OCCURRENCE OF INFLUENZA B IN SOUTHERN ENGLAND.

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SINCE the isolation of a virus by Smith, Andrewes and Laidlaw from patients suffering from influenza (1933), it has become increasingly obvious that only a part of the story was then revealed. For example, in an investigation of the 1939 epidemic in England (Stuart-Harris *et al.*, 1940) less than 30 per cent. of sera tested from patients with clinical influenza gave evidence of infection with the virus in question (now known as influenza A virus (Horsfall *et al.*, 1940). Recently Francis (1940) has described the isolation from an influenza outbreak in the U.S.A. of another type of virus which has been called influenza B virus. This virus, while producing the same range of symptoms as influenza A virus, is unrelated to it antigenically. Francis found that influenza due to virus B had been widespread in Eastern and South-eastern U.S.A. in January–February, 1940 ; further, study of sera preserved since 1936 showed that it had also occurred widely in an epidemic in California during that year.

During the winter of 1940–41 a mild outbreak of influenza occurred in Southern England. The results of an investigation of this epidemic have been published (Andrewes *et al.*, 1941), but although influenza A was probably not wholly responsible, no evidence of the presence of influenza B virus was obtained. The purpose of this paper is to show that influenza B virus was, however, present during the 1939 outbreak already mentioned.

MATERIAL AND METHODS.

Influenza B virus (Lee strain) was kindly sent to us by Dr. T. Francis through the International Health Division of the Rockefeller Foundation in the form of dried mouse lung. It had some 10 additional passages in mice here before use, but remained of rather low virulence for our mice ; unfiltered centrifuged suspensions of mouse lungs have therefore been used in our tests. The sera tested were obtained in January–March, 1939, and had been stored since then at — 2° C.

Serum neutralization tests were performed in the usual way, attention being directed to the presence or absence of a rise in antibody titre between the serum samples obtained at the onset of the illness and after about 10 to 14 days' convalescence. The end-point in a titration was taken as that serum dilution which reduced the lung lesions in a group of mice to 50 per cent. of the complete consolidation found in controls—the 50 per cent. maximum score end-point of Horsfall (1939).

RESULTS.

All the available sera were tested against influenza B virus, including those which were known to have shown a rise in titre against A virus ; the results

Tests of 1939 *Sera.*

Locality of outbreak.	Time of outbreak.	Result of test of sera for rise in antibodies to influenza A and B 1941.			
		Number tested.	Positive A.	Positive B.	Negative to A and B.
Warlingham Mental Hospital	February	3	0	2	1
Christ's Hospital, Horsham	,,	3	2	1	0
Lancing School	March	1	0	1	0
R.N. Chatham	January	3	0	1	2
,, ,,	February and March	15	2	0	13
H.M.S. " Wildfire," Sheerness	February	1	0	0	1
R.A.F. Eastchurch	January	1	0	1	0
,, ,,	March	7	3	2	2
H.M.S. " Ganges," Shotley	March and April	4	2	0	2
Totals		38	9	8	21

are summarized in the table. The rises in titre in the 8 B-positive cases were respectively 600-, 150-, 150-, 100-, 50-, 40-, 20-, and 10-fold.

It can be seen that not only was influenza B virus widespread during early 1939, but that it was found throughout the whole epidemic period, from January to March. It occurred in two of the institutions investigated at the same time as cases due to influenza A virus, and clinically was not distinguishable from virus A infection. No sera showed an antibody rise against both A and B, though such a finding suggestive of a double infection is recorded by Lennette *et al.* (1941).

It seems fair to assume that patients whose sera showed no rise in antibody against either A or B were suffering from infection with some agent other than those viruses. A-positive sera from Lancing and H.M.S. "Wildfire" were found in tests made in 1939, but were not available for test this year. Taking them into account, it appears that in eight of the nine outbreaks tested the etiology was complex—A and B both being present in 3, A and an unknown in 3, B and an unknown in 1, A, B and an unknown in the eighth. Lennette *et al.* (1941) have reported very similar findings to the above in epidemics in the West Indies and Eastern U.S. in the winter of 1940–41.

Clinically the B infections of 1939 were not distinguishable from those due to influenza A virus; they varied in type over the range met with in the epidemic as a whole. We failed in 1939 to find as much clinical difference as had been noted in the 1937 epidemic between cases due and not due to virus A infection. The finding now reported that influenza B virus was also prevalent in 1939 cannot be said to clarify the position fully at this stage; perhaps it is unreasonable to hope for clarification when we can only determine the presence of two out of the various agents probably concerned.

SUMMARY.

Evidence is produced that influenza B was present in England in January–March, 1939. Serological evidence indicates that outbreaks of clinical influenza occurring in a number of localities in that year were due in part to influenza A virus, in part to influenza B virus but, in more than half the cases, to neither virus.

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A MODIFICATION OF McINTOSH AND FILDES' ANAEROBIC TIN.

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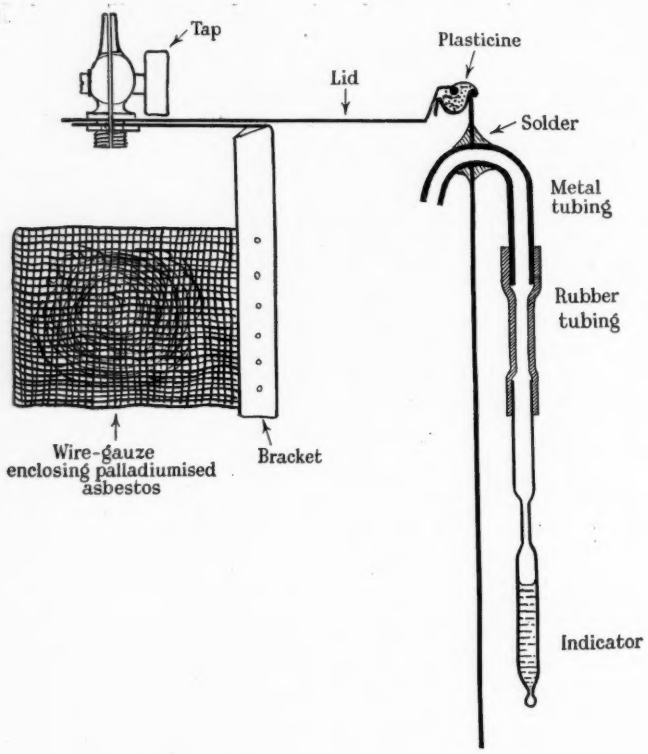
In 1916 McIntosh and Fildes introduced their anaerobe tin which revolutionized the methods of anaerobic culture. It consisted of a metal tin with a " lever-off " lid, in the centre of which was soldered a small gas-tight stopcock, to the underside of which was fixed a bracket holding a flat mass of palladiumized asbestos enclosed in an envelope of fine brass wire gauze. Anaerobic conditions were produced by heating the asbestos, replacing the lid, introducing hydrogen, and making the tin airtight by filling the gutter with plasticine. By this means, during the last years of the war of 1914–1918, all the anaerobes were grown successfully. In 1921 McIntosh and Fildes introduced an improvement on their original tin. This consisted of a glass jar with a carefully ground top on which was fitted a heavy brass lid carrying the palladiumized asbestos in an element which could be heated electrically ; the lid was also fitted with taps for evacuating the jar and filling it with hydrogen. The original anaerobe tin was largely given up for this more elaborate apparatus, which had the great advantage that the glass jar enabled one to see by means of an indicator tube that the anaerobic process was efficient. Owing to frequent breakages of the glass jar in many laboratories metal jars have been substituted which, whilst they have some of the advantages of the newer McIntosh and Fildes' apparatus, have the same disadvantage as the original tin in that the indicator tube cannot be seen, and so the perfection of anaerobic conditions cannot be checked until the jar in opened.

The original McIntosh and Fildes tin was an efficient piece of apparatus, and as it can be improvised in any laboratory at the cost of a few shillings it seems a pity that it should be forgotten and replaced by a much more elaborate apparatus which can only be obtained from a laboratory outfitter at a cost of many pounds. The chief drawback to the old apparatus was that it was quite easy to have a small leak which was not detectable until the tin was opened—which was too late.

In this article I propose to describe a modification of the original tin whereby an external indicator tube enables the worker to ascertain whether the anaerobiosis is effective. If this modification is used, then the original tin can be

used for anaerobic cultures of wounds with the same certainty of success as the more elaborate and expensive McIntosh and Fildes glass jar in common use, and with more certainty than the similar apparatus, almost as expensive, in use in many laboratories in which the glass jar has been replaced by one of metal.



The proposed modification is illustrated in the diagram. A 3-in. length of compo-tubing (external diameter about $\frac{3}{16}$ in.) is bent over at one end and this end is inserted into a hole punched in the side of the tin near the upper rim, and soldered in place to make the joint gas-tight. (Instead of the compo-tubing any right-angled length of solderable tubing can be used.) To the portion of the tubing extending down the outside of the tin a 2-in. length of stout rubber tubing is attached. The indicator solution (about 0·5–1·0 ml.) is placed in a $2\frac{1}{2}$ in. length of 6 mm. glass tubing which has been sealed at one end, and a constriction is made above the level of the fluid to avoid spilling; the open end is then attached to the rubber tubing.

The indicator solution which has been found most satisfactory is 1 per cent. glucose beef digest broth, adjusted to about pH 8·5–9·0, and coloured deep blue with methylene blue; this solution becomes bleached after about 2–3 hours' anaerobic incubation at 37° C. It may be preserved with 0·01 per cent. methiolate or 0·01 per cent. phenyl mercuric nitrate, to prevent growth of organisms and consequent destruction of the dye; the preservative has no effect on the efficiency of the indicator. Owing to saturation of water vapour inside the tin when in use there is no evaporation of the solution in the tube even after prolonged incubation; the tube may thus be left in place and the tin used repeatedly; when the colour change is eventually too small, owing to some destruction of the dye during each incubation, it is a matter of seconds to fit a tube of fresh indicator solution.

After the tin is made, and before it is put into use, McIntosh and Fildes advised that it should be varnished inside to prevent rusting by pouring in a little varnish, rinsing it around the tin, and pouring off the excess; also that the tin should be tested for leaks by placing 2 ml. of ether in the tin, pressing down the lid, and moulding the plasticine seal; the tap is then closed and the tin immersed in warm water, when a leak will be immediately detected.

Assuming that the tin is satisfactory, the same procedure is then followed as was described by McIntosh and Fildes in 1916. Since, however, this may not be familiar to the younger workers, a summary of their instructions may be useful:

1. Place cultures in the tin.
2. With a rag dipped in spirit spread the plasticine towards the periphery of the top of the tin.
3. Heat the capsule in bunsen flame.
4. Immediately force on the lid and push on to the open tap the rubber tubing from the hydrogen generator.
5. Turn on the gas.
6. Mould the plasticine with the spirit rag over the edge of the lid.
7. When the tin is quite cold (15–20 minutes) shut both taps and disconnect the generator, and place the tin in the incubator.

In the modified tin the external indicator tube shows by the decolorization of the dye that the anaerobic process is proceeding just as does a similar indicator tube in the glass jar of the McIntosh and Fildes newer apparatus. If the indicator solution does not lose its colour in a reasonable time, then there is a leak which must be remedied.

Comparison of the times taken for the indicator solution to lose its colour at various pH's in the modified tin and in the new McIntosh and Fildes glass jar.

Anaerobiosis was induced in a new McIntosh and Fildes glass jar and in a modified tin. Observations were made as to the time it took for the indicator tube to be decolorized. The results are set forth in the following table:

pH of glucose broth used for indicator solution.	Incubation time at 37° C.	Approximate percentage of colour persisting in—	
		Tin indicator.	Jar indicator.
7·3	6 hours	90 per cent.	90 per cent.
8·3	6 ,,	70 ,,	70 ,,
8·6	2 ,,	0 ,,	5 ,,
8·9	1½ ,,	0 ,,	10 ,,

These figures show that the tin reaches a state of complete anaerobiosis rather more quickly than does the jar, despite the fact that the external indicator on the tin communicates with the interior by several inches of tubing so that gaseous interchange is not so rapid as in the jar.

Many workers have shown that by means of the original tin all the anaerobic bacilli in wounds could readily be cultivated, and this I have confirmed in the past few months. The modification which I describe, of an indicator tube outside the tin, does not in the slightest degree increase the efficiency of the tin, but it does eliminate its most serious drawback—namely, that one did not know for certain if it had been perfectly sealed.

As many emergency laboratories are being equipped, and since laboratory apparatus is hard to come by, the suggested simple modification of the very cheap and efficient McIntosh and Fildes anaerobe tin is recommended. The modified tin may be obtained from Messrs. Baird & Tatlock (London), Ltd.

My grateful thanks are due to Prof. Alexander Fleming for much helpful criticism and advice in the preparation of this paper.

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EXPERIMENTS WITH RENIN.

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THE presence and characters of a renal pressor substance have been the subject of intermittent research since Tigerstedt and Bergmann (1898) first published their paper, and it has been established that there can be made from kidney substance an extract, renin, which on injection is capable of causing a transient rise in blood pressure.

We record here some experiments which show the effect of ether anaesthesia on the hypertension induced by such extracts. It is noticed also that there may be associated changes in the pulse rate and in the electrocardiograph.

METHODS.

Preparation of the Extract.

The method of extraction used was a modification of that described by Pickering and Prinzmetal (1938). In early batches their instructions were followed precisely; later a number of minor technical alterations were introduced and they were thought to improve the yield.

The modified method was as follows:

(1) The fresh rabbit kidney was decapsulated, sliced in half, and the pelvis and major portion of medulla snipped away. The remaining renal tissue, which for the most part was cortex, was minced and weighed.

(2) Chilled absolute alcohol was added, 2 c.c. for every gramme of the minced tissue, the whole thoroughly shaken and placed in a refrigerator for twelve hours. The supernatant alcohol was then drawn off and a fresh charge of chilled alcohol added. The mixture was returned to the refrigerator for a further twelve hours.

(3) The mixture was then filtered under pressure, the pulp being pressed down and washed through again with the filtrate. The pulp was then spread out and dried at a temperature of under 12° C.

(4) After a thorough drying the pulp was ground to a powder in a sterile mortar and weighed.

(5) Chilled Ringer solution was then added, 5 c.c. of solution to each 0·5 g. of dried powder, the mixture shaken gently and placed in a refrigerator for 24 hours.

(6) Final filtration was through glass-wool and muslin, the last drops being

squeezed out and added to the filtrate. This final filtrate comprised the extract used for the experiment.

The modifications introduced were: (1) Selection of the renal tissue, (2) double extraction of alcohol, (3) pressure filtration, (4) drying in the cold.

The dried powder (stage 4) kept at 0° C. was still suitable for extraction and use after 24 days. The saline extract tended to lose its potency slowly over 7 days.

A batch of the extract was used after shaking in the cold with acid washed Norite charcoal and another after treatment with colloidal zinc.

Extracts were similarly made from fresh human kidney, fresh rabbit muscle, rabbit heart and rabbit liver.

Blood Pressure Determinations.

Rabbits were prepared with carotid loops using the technique of Van Leersum (1911). Pressures were taken daily until constant readings were obtained; then, with a manometer in position, the extract was injected into an ear vein and pressures read every two minutes.

RESULTS.

Demonstration of Pressor Effects.

Rises of pressure of approximately 60 mm. of mercury were obtained after injections of 2–3 c.c. of the extract. The extract of fresh human kidney gave comparable pressor effects, but no increase in pressure was detected after the injection of Ringer solution nor with extracts prepared from rabbit muscle, heart or liver.

Kidney extracts which had been shaken with Norite charcoal or treated with colloidal zinc failed to show any pressor substances. As yet, we have not achieved recovery of the pressor substance from the charcoal or the precipitates.

The Influence of Anaesthesia.

Records were taken using the same batch of extract and the same rabbit, (A) without anaesthesia, (B) with anaesthesia induced before the injection and maintained up to the sixth minute following injection, (C) with anaesthesia induced two minutes after injection and terminated at the sixth minute. As an example of the results obtained, the records of one animal are given superimposed in Fig. 1.

It will be seen from the figure that injection of the extract is followed by a rapid rise of pressure recorded at two minutes; the pressure falls quickly for the next six minutes, and thereafter slowly returns to normal (A).

Induction of anaesthesia before injection prevents this rise (B).

If anaesthesia is delayed until the rise is established the blood pressure rapidly falls to normal levels, but the blood-pressure effect shows itself again when anaesthesia is discontinued (C).

Electrocardiographic Findings.

While taking the carotid pressure after injection of the extract the slowing and irregularity of the pulse were detected. Confirmation of these observa-

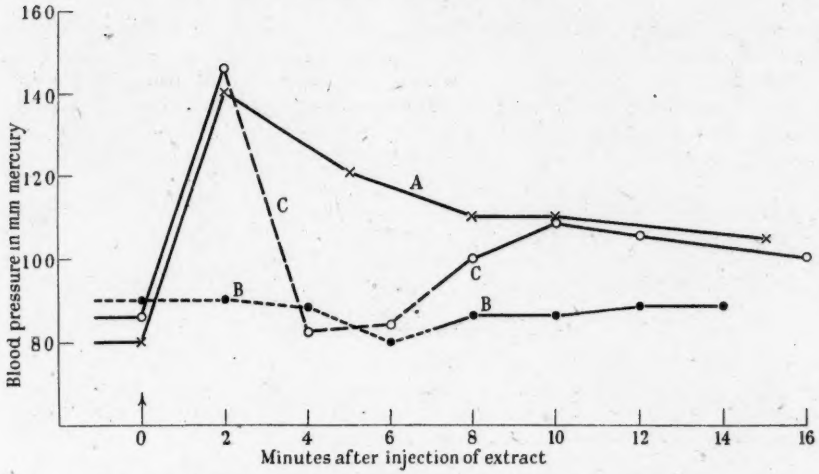

FIG. 1.—Blood-pressure records of Rabbit 958 on three successive days:
A. ——×—— 2½ c.c. kidney extract intravenously.
B. ——●—— 3 c.c. ,, ,,
Anaesthesia for first 6 mins.
C. ——○—— 3½ c.c. kidney extract intravenously.
Anaesthesia 2–6 min.
Anaesthesia is indicated by broken lines.

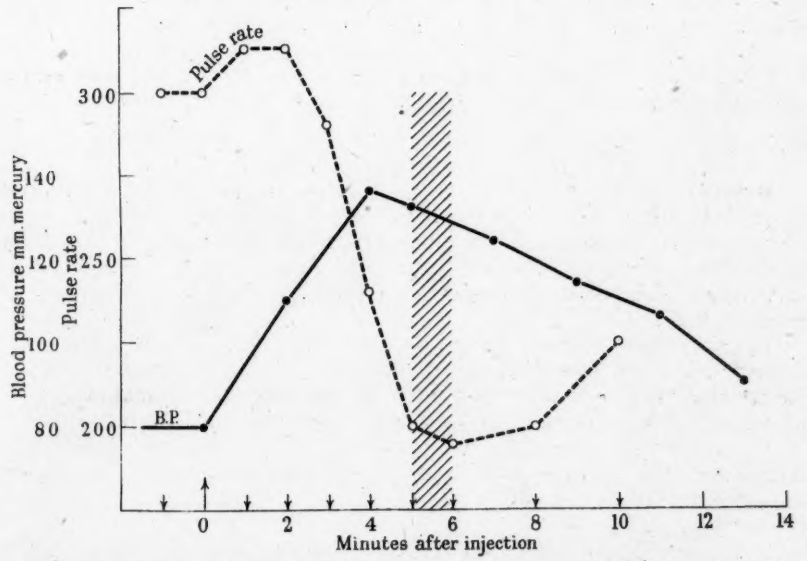

FIG. 2.—Rabbit 1016. Blood pressure and pulse rate (broken line) after 3 c.c. of extract. Shaded area represents period over which extrasystoles were recognized. Small arrows = electrocardiographs.

tions was attempted by means of serial electrocardiographs taken following injection of the extract. They demonstrated slowing of the pulse which was most marked when the rise of blood pressure was at its height and also ventricular extrasystoles round about the peak of the pressure. A chart showing these findings is given in Fig. 2, and in Fig. 3 are given the first three and the last three of the electrocardiographs taken.

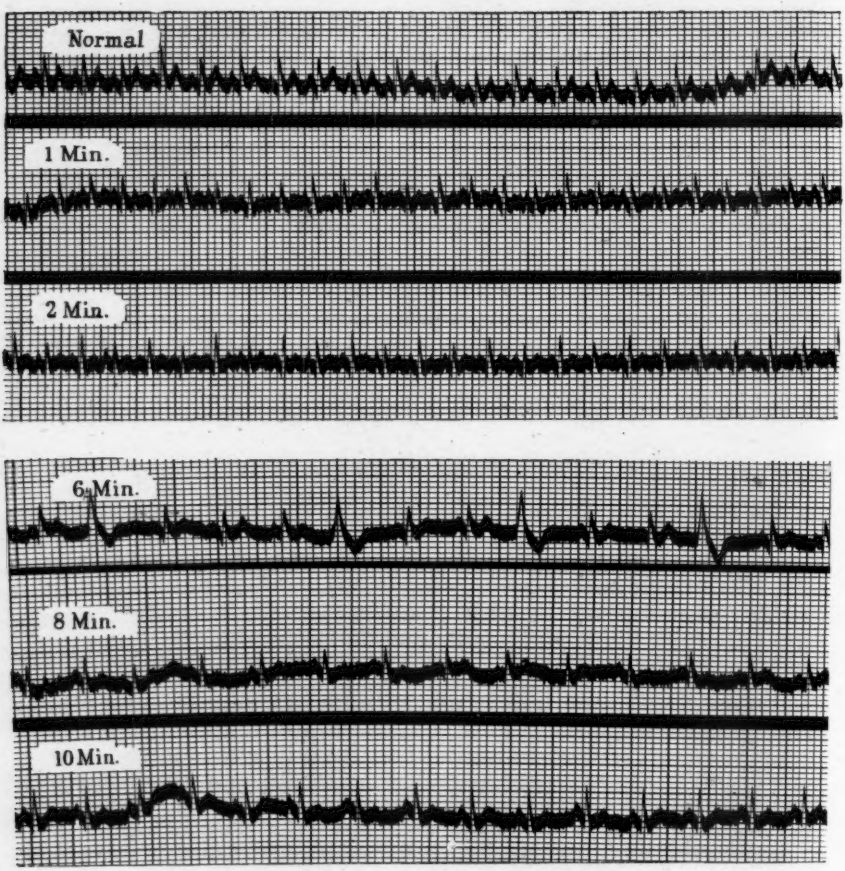

FIG. 3.—First three and last three electrocardiographs of Rabbit 1016 (see Fig. 2).

DISCUSSION.

Working on various hypotheses, numerous attempts have been made to induce hypertension in animals in the hope that some light may be thrown on the pathology of the human condition.

The frequent association of atheroma with hypertension indicated the possibility that essential hypertension was compensatory to atheromatous obstruction in the vessels of some vital organ. It was found, however, that there were no significant changes in blood pressure in animals in which an advanced degree of atheroma had been experimentally produced (Dominguez, 1927 ; Scarff, 1927).

The part played by the carotid sinus in the regulation of the blood pressure seemed to offer a likely field for exploration, and it was found that denervation of the carotid sinuses led to a persistently raised blood pressure in many kinds of animal (Koch, 1931 ; Heymans, Bouckaert and Regniers, 1933). This rise in pressure was not abolished by anaesthesia, and animals which survived any length of time showed a high incidence of vascular lesions (Kremer, Wright and Scarff, 1933).

Because of the occurrence in man of hypertension secondary to a kidney lesion, the possibility of a renal origin for hypertension has always been prominent, although the original work of Tigerstedt and Bergmann for a long time did not receive the attention it deserved.

The effects on the blood pressure of various forms of kidney damage have been extensively studied ; the types of injury have included partial infarction, unilateral and bilateral nephrectomy, glomerular embolism and oxalate nephritis. In all cases the results have been negative or conflicting (Scarff and McGeorge, 1937). Goldblatt (1932, 1940) revived interest in this line of research by showing that partial obstruction of the renal circulation produced a sustained rise in blood pressure. This stimulated work on renal ischaemia, and also re-awakened interest in the pressor substances found in the kidney.

In the work on experimental hypertension the effects of general anaesthesia on the raised blood pressure have been too little stressed, and it seems certain that much information on blood pressure has been lost by relying on " acute " experiments conducted throughout under full general anaesthesia. Apart from this a study of the effect of anaesthesia on the blood pressure may be informative. The experiments detailed here confirm the findings of Pickering and Prinzmetal (1938) and of other workers that a pressor substance can be extracted from kidney substance and that its action can be prevented by ether anaesthesia. Furthermore, it is shown that even when the rise in blood pressure has been established by its action, ether anaesthesia will rapidly reduce it to a normal level ; also the continuance of the pressor action is demonstrable if the anaesthesia is discontinued within the normal time of action of the substance. We consider that this behaviour under anaesthesia shows that the mechanism of the induction of hypertension by renin must differ essentially from the mechanism involved in carotid sinus denervation.

In general the findings favour the view that renin acts through the central nervous system, but direct action on the peripheral blood vessels cannot be excluded.

SUMMARY.

The abolition by ether anaesthesia of the pressor action of renin in rabbits is confirmed.

It is also shown that the induction of ether anaesthesia will rapidly lower

to a normal level a blood pressure raised by this substance, but that the effect of the previous injection of renin is demonstrable if the anaesthesia is discontinued.

The rise in blood pressure is accompanied by slowing of the pulse rate and frequently by the appearance of extrasystoles.

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